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IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	Zoltan GREFF et al		
Patent App.	10/030,436		
Filed	21 March 2002	Conf. No. 6522	
For	2,3-BENZODIAZEPINE DERIVATIVES		
Art Unit	1624	Examiner COLEMAN, B	

Hon. Commissioner of Patents
Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132

I, Laszlo G. Harsing, a citizen of Hungary, residing at 116 Bokenyfoldi út, 1165 Budapest, Hungary, declare as follows:

THAT I have a number of years of experience in the preparation and testing of pharmaceutically active compounds in the treatment of neurodegenerative disorders;

THAT my full curriculum vitae may be attached thereto;

THAT I am an Applicant in U.S. Patent Application Serial No. 10/030,436 filed 21 March 2002 and directed to 2,3-BENZODIAZEPINE DERIVATIVES;

THAT in order to establish that the present application enables one „skilled in the art" to use the compounds of the Formula (I) to treat Parkinson's disease and multiple sclerosis in mammals, including humans, I have either personally conducted or supervised the carrying out of the following tests:

1 Effects of AMPA antagonists in an animal model of Parkinson's disease

The clinical rating scale of Parkinson's disease consists of several measures of parkinsonian features (tremor, posture, catalepsy, bradykinesia, gait, balance and defense reaction). The rodent model of Parkinson's disease used was a drug-induced monoamine-depletion, which reduces striatal dopamine content by about 95%, and produces a cataleptic effect, which is also a characteristic of human Parkinson's disease (Cooper DR. et al. (1987) L-dopa esters as potential pro-drugs: behavioral activity in experimental models in Parkinson's disease. J. Pharm. Pharmacol. 39, 627-635).

1.1. Methods

Male Wistar rats weighing 210-225 g were depleted of brain monoamine by pretreatment with reserpine (5 mg/kg in 2 ml/kg volume, intraperitoneally) 1 hour before the experiment. Then, 1 hour later, 3 dose levels (2.5 mg/kg, 5 mg/kg, 10 mg/kg in 5 ml/kg volume orally for the Compound of Example 27 and 3.75 mg/kg, 7 mg/kg and 15 mg/kg for bromocriptine) of test or reference substance were administered to separate groups of rats and an additional group was treated with vehicle. Each experiment group consisted of 8 rats. Catalepsy was evaluated with a 12 cm long and 10 cm high horizontal bar at every hour for 5 hours. Each rat was tested with respect to its behavior on the bar. The rat gained a „+" score if it maintained the abnormal (postural) position for more than 30 seconds. In case catalepsy was reduced by more than 50% in any of the treated groups compated the reserpine+vehicle treated group, an ED_{50} was calculated using the method of Litchfield and Wilcoxon.

1.2. Results

According to results presented in Table 1 below, the compound of Example 27 of the present Patent Application reduced reserpine-induced catalepsy with similar efficacy to bromocriptine. The results suggest that compounds of Formula (I) of the present invention can be suitable for the treatment of Parkinson's disease. This suggestion is strenghtened by previous publications which clearly demonstrated that non-competitive AMPA receptor antagonists had strong antiparkinsonian effect in a primate model of the disease (Konitsiotis, S. et al. (2000) AMPA receptor blockade improves levodopa-induced dyskinesia in MPTP monkeys. Neurology 54, 1589-1595).

Table 1

Antiparkinsonian effect of AMPA antagonists as assessed by improvement of reserpine-induced catalepsy in rats

Compound	ED_{50} (mg/kg p.o.)
Bromocriptine (reference compound)	3.0
Compound of Example 27	4.4

2 Protection against inflammation induced by experimental autoimmune encephalomyelitis (EAE) in rats

Multiple sclerosis is an autoimmune disease of the central nervous system that results in progressive fall of sensory and motor functions due to destruction of the myelin sheet of axons leading to neuronal death. It has been clearly shown that glutamate receptors including AMPA receptors are present in oligodendrocytes and these cells are highly sensitive to glutamate-induced excitotoxicity (Matute, C. et al. (2001) The

link between excitotoxic oligodendroglial death and demyelinating diseases. Trends Neurosci. 24, 224-230). Experimental autoimmune encephalomyelitis (EAE) can be induced in laboratory animals by treating them with basic myeloprotein or its fragments carrying the antigen moiety. EAE mimics the basic characteristics of the human disease and can be regarded as an acceptable model of multiple sclerosis (Smith, T et al.: (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. Nat. Med. 6, 62-66).

2.1 Methods

The experiments were performed according to the slightly modified method of Smith and al (Smith, T et al. (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. Nat. Med. 6, 62-66). Lewis rats (230-270 g, Charles River Hungary) were immunized subcutaneously in the dorsal surface of each hind paw with 50 µl inoculum containing 100 µg of guinea pig myelin basic protein that was emulsified in Freund's complete adjuvant containing 5.5 mg/ml Mycobacterium tuberculosis H37a (Difco). The compounds were administered intraperitoneally twice a day for 7 days starting on the day 10 after immunization. The dose of the compound of Example 28 was 3 mg/kg i.p. twice a day and the dose of the reference compound {(±)-7-acetil-5-(4-aminophenyl)-7,8-dihydro-8-methyl-9H-1,3-methylenedioxy[4,5-h][2,3]benzodiazepine, GYKI53405} was 10 mg/kg i.p. twice a day. On day 17 the rats were anesthetized with sodium pentobarbital at 60 mg/kg i.p. and perfused via the heart with a fixative solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline. Samples were embedded in paraffin. Transversal sections (6 µm thick) of the lumbar spinal cord and telencephalon were cut and stained with Luxol Fast Blue (LFB).

2.2. Results

Key histological features of experimental autoimmune encephalomyelitis were multifocal perivascular inflammation in the medulla and spinal cord, gliosis, demyelinisation and necrotic or apoptotic degeneration of glial cells or neurons. According to results presented in Table 2 below, compound of Example 28 fully prevented all inflammatory consequences of experimental autoimmune encephalomyelitis in rats. GYKI53405, the reference compound afforded smaller protection under the same experimental conditions. These results clearly suggest that compounds of Formula (I) of the present invention can be suitable in the treatment of multiple sclerosis which possibly is in keeping with observations demonstrating a beneficial effect of non-competitive AMPA antagonists in animal models of multiple sclerosis (Smith, T et al.: (2000) Autoimmune encephalomyelitis ameliorated by AMPA antagonists. Nat. Med. 6, 62-66).

Table 2

Improvement of histological signs of experimental autoimmune encephalomyelitis by treatment with AMPA receptor antagonists in rats

Histological findings	Control		GYKI53405		Compound of Example 28	
	Mean score	Rate	Mean score	Rate	Mean score	Rate
Medulla oblongata						
multifocal perivascular inflammation in the medulla	2,0	6/6	0,2	1/5	0,0	0/5
gliosis	2,0	5/6	0,2	1/5	0,0	0/5
necrotic/apoptotic cells (glial cells and/or neurons)	0,7	3/6	0,0	0/5	0,0	0/5
Spinal cord						
multifocal perivascular inflammation	2,2	6/6	0,8	2/5	0,0	0/5
demyelinisation	1,5	6/6	0,8	2/5	0,0	0/5
gliosis	1,8	6/6	0,8	2/5	0,0	0/5
necrotic/apoptotic cells (glial cells and/or neurons)	2,0	6/6	0,4	2/5	0,0	0/5
Nerves						
demyelinisation	1,8	5/5	0,0	0/3	0,0	0/5
glial destruction	1,8	5/5	0,0	0/3	0,0	0/5

THAT based upon the experimental data presented above, I conclude the following:

The compounds of the Formula (I) of the present invention are non-competitive AMPA antagonists. Experimental results obtained from animal model experiments indicate that compounds of the present invention possess strong neuroprotective effect, which is useful in the treatment of conditions and diseases of the central nervous system. Besides their effect in animal models of epilepsy, stroke, amyotrophic lateral sclerosis and cystic periventricular leukomalacia, the compounds according to the present invention demonstrate activity in animal models of Parkinson's disease and multiple

sclerosis as well. All these activity of the compounds of Formula (I) of the present invention are believed to be resulting from AMPA receptor antagonism. The mechanism whereby AMPA receptor antagonists prevent neuronal cell death in disorders of very different etiology is the inhibition of glutamate-induced excitotoxicity, which is a major mechanism leading to apoptosis and necrosis of nerve cells.

The experimental data presented hereinabove provide support for the establishment of utility of the compounds according to the present invention in neurodegenerative disorders.

In summary, the compounds of the Formula (I) according to the present invention have suprisingly advantageous pharmacokinetic and metabolic properties which result in a preferable pharmacological and toxicological profile.

THAT I am aware of no information inconsistent with that presented above or which would lead one to a contrary conclusion;

and

I further declare that all statements made herein of my own are true and that all statements made on information and belief are believed to be true; and further

THAT these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issues thereon.

Dated:________________ Signed:________________________

Laszlo G Harsing, MD,PhD, DSc

CURRICULUM VITAE

Name: Dr. Laszlo Gabor Harsing

Date and Place of Birth: September 9, 1947, Budapest, Hungary

Citizenship: Hungarian

Marital Status
Married, Elizabeth Veinperl-Harsing
Pharmacist

Present Employment
Vice Director, Head of Division
Division of Preclinical Research
EGIS Pharmaceuticals Ltd

Education
1966: Graduated from secondary school

1972: Medical degree obtained with qualification of "Summa cum Laude" at the Semmelweis Medical School, Budapest

1984: Ph.D. degree obtained in neuropharmacology
Thesis: Regulation of Cholinergic Neurotransmission in the Striatum
Hunagrian Academy of Sciences, Budapest

1992: Doctor of Sciences (D.Sc.) degree obtained in neuropharmacology
Thesis: The Role of Heterogenous Alpha-2 Adrenoceptors in the Regulation of Noradrenergic Neurotransmission
Hunagrian Academy of Sciences, Budapest

1994: Lecturer in Pharmacology, degree obtained at the Department of Pharmacology, Semmelweis Medical School, Budapest

Employment in Hungary
1968-1972: Teacher in Physiology, Department of Physiology, Semmelweis Medical School, Budapest

1972-1981: Assistant Professor of Pharmacology, Department of Pharmacology, Semmelweis Medical School, Budapest

1981-1986: Research Fellow and Senior Research Fellow, Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest

1986-1995: Associate Professor of Pharmacology, Department of Pharmacology Postgraduate Medical School, Budapest

1992-2000: Head, Department of Neurobiochemistry
Vice Director of Biological Research
Institute for Drug Research, Ltd., Budapest

2000-at present: Vice Director and Head
Division of Preclinical Research
EGIS Pharmaceuticals Ltd

Employment in the United States of America
1980-1981: Visiting Scientist, Fogarty International Fellowship, Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Washington, D. C., USA, Head: Dr. Erminio Costa, 20 months

1984-1985: Visiting Fellow, Department of Anesthesiology, Albert Einstein College of Medicine, New York, NY, USA, Head: Dr. Derick D. Duncalf, 13 months

1989-1992: Research Scientist, Center for Neurochemistry, The Nathan S. Kline Institute for Psychiatric Research, Rockland Psychiatric Center, Orangeburg, NY, USA, Head: Dr. Abel Lajtha, 36 months

1995-1996: Visiting Research Scientist, Fogarty International Fellowship, Department of Neuroscience, University of Pittsburgh, Pittsburgh, PA, USA, Head: Dr. Michael J. Zigmond, 20 months

Short-Term Fellowships
1975: Department of Pharmacology, 1st Medical University, Moscow, USSR

1976: Institute of Physiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

1977: Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland

1982: National Institute of Mental Health, Washington, D.C., USA

1987 Department of Pharmacology, University of Geneva, Geneva, Switzerland

1988 Universidad Central de Venezuela, Caracas, Venezuela

1993 Nathan Kline Institute for Psychiatric Research, Orangeburg, NY, USA

1998 Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA

Research Activity
Publications in journals: 93

Chapters published in textbooks: 37

Abstracts appeared in journals: 26

Abstracts presented in scientific meetings: 95

Number of citation: 1170, January 2003

Cumulative impact factor of publications: 206.470, January, 2003

Teaching Experience
1970-1972: Teaching of physiology for medical students

1972-at present: Teaching of pharmacology for medical students and in postgraduate courses

Research Interest
Pharmacology, Neuropharmacology, Neurochemistry, Neurotransmitter release and interactions, Pharmacology of Transporters

Experimental Procedures Employed
Brain slice techniques, Isolated organs, Microdialysis and HPLC analysis, Measurement of radiolabeled and endogenous neurotransmitter release

Membership
Society for Neuroscience, USA
British Pharmacological Society
European Society for Neurochemistry
Hungarian Pharmacological Society
Hungarian Physiological Society

Journal Editorial Advisory Board, Member at
Neurochemical Research
Neurochemistry International

Honour
Bela Issekutz Lecture and Medal
Budapest, 2001

Reference
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Dr. Laszlo G. Harsing, Jr.

LIST OF PUBLICATIONS

1. RESEARCH ARTICLES

1. Hársing, L., Hársing, L., Jr., Bartha, J.: Uj szempontok az intrarenális haemodynamikában. Orvostudomány, 1972, 23, 295-301.

2. Kover, G., Harsing, L. G., Harsing, L.: Effect of elevated renal venous pressure on intrarenal haemodynamics. Acta Physiol. Hung., 1974, 45, 173-180.

3. Knoll, J., Makleit, S., Friedmann, T., Harsing, L. G., Jr., Hadhazy, P.: Circulatory, respiratory and antitussive effects of azidomorphine and related substances. Arch. Internat. Pharmacodyn., 1974, 210, 241-249.

4. Knoll, J., Makleit, S., Friedmann, T., Hársing, L. G., Hadházy, P.: Az azidomorfin és származékainak hatása a keringésre, légzésre és köhögésre. Orvostudomány, 1975, 26, 89-95.

5. Harsing, L. G., Jr., Bartha, J., Harsing, L.: Effect of carotid occlusion on intrarenal haemodynamics. Acta Medica Hung. 1976, 33, 371-378.

6. Knoll, J., Hársing, L. G., Friedmann, T.: A 3-éter-6-azidomorfinok farmakológiája. Az azidoetilmorfin egy uj köhögéscsillapitó szelektálása. Orvostudomány, 1976, 27, 263-284.

7. Knoll, J., Harsing, L. G., Jr., Friedmann, T.: Azidoethylmorphine, a new potent non-narcotic oral antitussive. Acta Physiol. Hung., 1977, 50, 341-356.

8. Vizi, E. S., Ronai, A. Z., Harsing, L. G., Jr., Knoll, J.: Inhibitory effect of dopamine on acetylcholine release from caudate nucleus. Pol. J. Pharmacol. Pharmac., 1977, 29, 201-211.

9. Vizi, E. S., Harsing, L. G., Jr., Knoll, J.: Presynaptic inhibition leading to disinhibition of acetylcholine release from interneurons of the caudate nucleus: effect of dopamine, beta-endorphin and D-Ala2-Pro5-enkephalinamide. Neuroscience, 1977, 2, 953-961.

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11. Harsing, L. G., Jr., Magyar, K., Tekes, K., Vizi, E. S., Knoll, J.: Inhibition by deprenyl of dopamine uptake in rat striatum: a possible correlation between dopamine uptake and acetylcholine release inhibition. Pol. J. Pharmacol. Pharmac., 1979, 31, 297-307.

12. Harsing, L. G., Jr., Illes, P., Furst, S., Vizi, E. S., Knoll, J.: The effect of prostaglandin E1 on acetylcholine release from cat brain. Acta Physiol. Hung., 1979, 54, 177-185.

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14. Vizi, E. S., Harsing, L. G., Jr., Zsilla, G.: Evidence of the modulatory role of serotonin in acetylcholine release from striatal interneurons. Brain Res., 1981, 212, 89-99.

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met5-enkephalin and beta-endorphin hypothalamic content in rats receiving anorectic drugs: differences between d-fenfluramine and d-amphetamine. Neuropharmacology, 1982, 21, 141-145.

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17. Harsing, L. G., Jr., Yang, H.-Y. T., Costa, E.: Accumulation of hypothalamic endorphins after repeated injections of anorectics which release serotonin. J. Pharm. Exp. Ther., 1982, 223, 689-694.

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53. Harsing, L., G., Jr., Vizi, E. S.: Alpha-2 adrenoceptors are not involved in the regulation of striatal glutamate release: comparison to dopaminergic inhibition. J. Neurosci. Res., 1991, 28, 376-381.

54. Kiem, D. T., Bartha, L., Harsing, L. G., Jr., Makara, G. B.: Reevaluation of the role of alpha-2 adrenoceptors in morphine-stimulated release of growth hormone. Neuroendocrinology, 1991, 53, 516-522.

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56. Sershen, H., Harsing, L. G., Jr., Banay-Schwartz, M., Toth, E., Hashim, A., Ramacci, M. T., Lajtha, A.: Effect of acetyl-l-carnitine on the dopaminergic system in aging brain. J. Neurosci. Res., 1991, 30, 555-559.

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58. Harsing, L. G., Jr., Sershen, H., Lajtha, A.: N-type Ca^{2+} channels are involved in the dopamine releasing effect of nicotine. Neurochemical Research, 1992, 17, 729-734.

59. Sershen, H., Hashim, A., Harsing, L., Lajtha, A.: Ibogaine antagonizes cocaine-induced locomotor stimulation in mice. Life Sci., 1992, 50, 1079-1086.

60. Harsing, L. G., Jr., Sershen, H., Lajtha, A.: Dopamine efflux from striatum after chronic nicotine: evidence for autoreceptor desensitization. J. Neurochem., 1992, 59, 48-54.

61. Harsing, L. G., Jr., Sershen, H., Toth, E., Hashim, A., Ramacci, M. T., Lajtha, A.: Acetyl-L-carnitine releases dopamine in rat corpus striatum: an in vivo microdialysis study. Eur. J. Pharmacol. 1992, 218, 117-121.

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6. INVITED LECTURES

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